Methods for Selective Enrichment of Campylobacter spp. from Poultry for use in Conjunction with DNA Hybridization

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Running Head: Campylobacter spp. DNA probe

ABSTRACT

A DNA hybridization test initially described for use with human fecal specimens was investigated for application to the detection of *Campylobacter* spp. in poultry samples. The test chemistry involves solution phase hybridization and detection by means of an enzymatically generated colorimetric endpoint. DNA probes used in the test system are targeted to unique sequences of ribosomal RNA and are specific for *C. jejuni*, *C. coli*, *C. laridis* and *C. fetus* subsp. *fetus*. Initial experiments with pure cultures of *C. jejuni* established the sensitivity limit of the DNA hybridization assay at approximately 10^{6-7} cfu/sample. Experiments were designed to define optimal conditions for recovery and selective enrichment of *Campylobacter* spp. from chicken carcasses for use in conjunction with the DNA hybridization. Following overnight enrichment, cultures were swabbed onto Campy-Cefex plates and allowed to incubate for 24 h. This overnight growth was then suspended and assayed with the DNA probe. The remainder of the overnight enrichment was centrifuged and the resulting pellet was analyzed. Thirty-eight chicken carcasses were assayed for *Campylobacter* spp. by DNA probe and cultural methodology using enrichment culture and selective plating. Cultural procedures isolated *Campylobacter* spp. from 23 carcasses, while the DNA probe detected the organism from 21 carcasses. The DNA probe registered 5 "false" positives and 7 "false" negatives relative to the cultural bacteriologic approach.

INTRODUCTION

The Centers for Disease Control estimates that *Campylobacter jejuni* infections in humans occur at the rate of 2.2 million per year (*7*). Tauxe, *et al*. (*6*) found that 70% of the cases were related to consumption of underprocessed chicken carcasses. The vast majority of *C. jejuni* infections are sporadic, with only limited numbers of outbreaks occurring. Other *Campylobacter* spp. are also implicated as causative agents of disease.

This strong evidence of disease association with a food product makes it incumbent upon food services to ensure the absence of *Campylobacter* spp. in ready to consume food products. Cultural methods exist for the isolation and identification of *Campylobacter* spp. in food (*4*). Testing for this causative agent in foods has been limited or, nonexistent, in both governmental regulatory facilities and in industrial quality control laboratories. Three possible explanations exist: 1) There is indifference on the part of regulatory agencies and the food industry, 2) resources are too limited to allow for testing of significant human enteropathogens, or 3) methods are too cumbersome or inadequate to warrant testing. The first possibility is unlikely, however, the second and the third possibilities may serve as an explanation. A simple, inexpensive, and reliable analytical method for detecting the presence of *Campylobacter* spp. in foods is required.

The purpose of the following experiments was to compare bacteriologic cultural methods with a DNA probe procedure.

MATERIALS AND METHODS

PHASE I:

The intestinal contents from *Campylobacter* spp.-free chickens, held in isolation units, were diluted 1:10 (w/v) in a buffered peptone suspension. A 1:1 dilution of fecal cultures with suspensions of 0, ca. 10^6, ca. 10^7 and ca. 10^8 colony forming units (CFU) of two *C. jejuni* poultry isolates was made. DNA probe analysis was carried out on these suspensions as described below. The organisms were grown overnight at 42^{o}C in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) on Campy-Cefex medium (3) before suspending the growth to the appropriate optical density. The suspensions were enumerated by plating decimal dilutions onto brucella agar (Difco, MI) and incubating 24 h at 42^{o}C in a microaerobic atmosphere.

Nucleic acid hybridization procedures for detecting the homologous *Campylobacter* spp. ribosomal nucleic acid sequences are detailed in the GENE TRAK product literature. Positive control and Negative control samples were tested together with the test samples. All samples suspensions (described above) were added to a clean test tube in the amount of 0.20 ml. Seven-tenths ml of a lysis solution (guanadinium thiocynate; *1*) was added to each tube and the rack was shaken, by hand, for 5 seconds. The *Campylobacter* spp. DNA probe solution was mixed, and 0.10 ml of the solution was added to each test tube. The dipstick, which provided the solid phase for the reaction, was placed into the tubes. The materials were mixed and incubated together with the dipstick for one hour at room temperature. After this incubation period, the dipstick was washed in wash solution (*1*) and blotted on absorbent paper. The dipstick was then placed into a second tube containing a solution of enzyme conjugate and incubated at room temperature for 20 minutes. After the 20 minute incubation period, the dipstick was removed from the test tube and washed at room

temperature, and then blotted. To a third test tube, 0.75 ml of the substrate-chromagen was added. The dipstick was placed into the third test tube and incubated at room temperature for 30 minutes. After this incubation period, the tube was read in a GENE-TRAK photometer. The Negative control tube was read against a blank tube. The Positive control tube was also read against the blank tube. The sample absorbance values were read against a the Negative Control tube. The absorbance value obtained for the Negative Control needed to be equal to or below 0.15 for the assay to be valid. The absorbance value obtained for the Positive Control needed to be equal to or above 1.00. Samples which produced an absorbance value less than or equal to 0.10 indicated an absence of *Campylobacter* spp., while those with values greater than 0.10 indicated the presence of *Campylobacter* spp.

PHASE II:

Six fresh, chilled, whole chicken broiler carcasses were returned from the retail market to the laboratory within 15 min, and held under refrigeration at $4^{O}C$ until laboratory analysis. Individual carcasses were transferred to gallon size, self-sealing plastic bags (Ziplock, Dow; Indianapolis, IN), and 200 ml of sterile buffered peptone added to the contents. The bags were sealed and the contents of the bags shaken and massaged for two min. The rinse, buffered peptone, contained the representative, carcass-associated microflora. This bacterial suspension was passed through a cheesecloth to remove the associated fat globules and debris. The cheesecloth filtered sample was centrifuged at 10,000 x g for ten min and the supernatant fluid discarded. The pellet was resuspended in 2 ml of buffered peptone. These materials were held on ice until assaying for *Campylobacter* spp. by both cultural and DNA probe methodology, as described below (less than 4 hours after purchase). To these samples (from the first four carcasses) 1 ml bacterial suspensions was added containing ca. 10^{2-3} CFU *C. jejuni* (B-41).

Samples from the next two carcasses contained only indigenous *C. jejuni*. Each of these samples were processed as indicated in Table 1. There were 11 sample "site"s for both the cultural and DNA probe methodologies which were assayed for *Campylobacter* spp. during each carcass analysis. These "points" were designated at applicable locations throughout the cultural procedures. The enrichment methodology and the description of the selective/differential Campy-Cefex media are published elsewhere (*3,4,5*).

PHASE III:

After data were gathered from Phase II, we decided to include only sample points #10 and #11 This involved testing the DNA probe against (A) the overnight, centrifuged enrichment broth and (B) the suspension of growth from a selective plate which was swabbed with the original overnight enrichment culture that had also been incubated overnight. Concomitantly, the centrifuged, 2 ml resuspended carcass wash-bacterial suspension, and the centrifuged enrichment cultures was swabbed and streaked onto Campy-Cefex and incubated as described above. In total, 38 retail market, whole broiler carcasses were analyzed for the comparison.

RESULTS

Data gathered in Phase I of the experiment are shown in Table 2. The probe would, generally, detect the organism when *C. jejuni* (in pure culture) was present at levels of 10^6 and higher. Slightly higher absorbances were observed with pure culture as compared to the absorbances detected with Campylobacter and mixed cultures (feces). Usually, when the organism was present at levels of 10^7 among the more numerous fecal flora, the DNA probe could detect the organism.

Table 3 contains data which indicate the absorbances associated with the 13 designated sampling "points." Among those carcass samples inoculated with *C. jejuni*, sample points #10 and #11 were consistent in identifying the contaminated specimens. Sample "points" #10 and #11 also yielded positive signals from the uninoculated carcass samples. With few exceptions, the other sample points were limitedly successful in identifying the presence of inoculated *C. jejuni* among the carcass associated flora.

Table 4 illustrates the results obtained in the analysis of carcasses for *Campylobacter* spp. by both bacteriological culture and by DNA probe technologies. Sample "points" #10 and #11, only, were used for assay. Cultural procedures isolated *Campylobacter* spp. in 23 of the 38 carcasses sampled. The DNA probe technology yielded positive signals for *Campylobacter* spp. in 6 of 38 carcasses at sample site #11 and in 16 of 30 carcasses at sample site #10. The combination of points #10 and #11 yielded 21 positive signals out of the 38 carcasses sampled. The gene probe system registered 5 positive signals where the cultural data did not yield colonies of *Campylobacter* spp. among the 38 carcasses. The gene probe system did not register 7 carcasses "positive" while the cultural methodology did isolate the organism. In total, the combination of the gene probe and the culture methodology indicated the presence of *Campylobacter* spp. in 28 of 38 carcasses sampled.

DISCUSSION

In Phase I, we compared the absorbance signals detected with and without chicken fecal flora. We made this comparison for two reasons. The first reason was that we could maintain chickens in isolation units, test, and confirm the absence of *Campylobacter* spp. in the bird feces. The second reason was that the fecal flora may contribute significantly to the carcass associated flora after processing. Due to the high frequency of contamination, there is great difficulty in obtaining *Campylobacter* spp.-free chicken carcasses. Consequently, detection among such high numbers of non-Campylobacter organisms would be a most stringent test. Levels of greater than 10^{11}/g of feces can be expected, and the capacity of the system to recognize the presence of *Campylobacter* spp. in this system should be considered as more rigorous than detection of the organism on a food product. Because the comparison in the presence of feces vs. pure culture was so extreme, the information gathered supports the system as highly selective, and competing microflora do not substantially detract from the positive signal.

Phase III served to evaluate the effectiveness of the gene probe in identifying the presence of *Campylobacter* spp. from among the flora of the poultry carcass. It is appreciated that by agitating carcasses more rigorously, or for longer durations (*2*), a greater frequency of the target organism may be expected. Indeed, more diligent approaches can always yield greater frequency of *Campylobacter* spp. (*4*). Whenever multiple approaches are used to evaluate the presence of an enteropathogen in association with a food, or even simply repeating the same approach several times, the increasing frequency of the organism can almost be guaranteed. This is much analogous to the increasing sensitivity of analytical chemical instruments which are now capable of detecting

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diminishingly small quantities of any molecule. It is not certain that the "false" positives detected in this experiment were, indeed, truly false. What is clear is that this DNA probe system adds an additional, useful analytical tool for bacteriologist to consider when assaying foods for the presence of *Campylobacter* spp.

The criteria for Positive and Negative controls were not always met in our assays. In our pure culture tests (Table 2), we would have observed several more "positive" signals with the DNA probe if the criterion for detection was not so stringent. As such, modifying these criteria could yield more sensitive and accurate identification of samples adulterated with *Campylobacter* spp. As this system is still in its developmental stages, further modifications are likely before the product has realized its ultimate value. The main advantage of such a system is in its capacity to take subjective technician evaluation out of bacteriological assays.

Acknowledgement

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Table 1. Sampling points used during the bacteriological culture and GENE-TRAK DNA probe analysis of chicken carcasses for detection of *Campylobacter* spp.

Time (h)	Procedure
0	Wash carcass in 200 ml PBS
0	centrifuge, resuspend in 2 ml PBS--#1 0.2 ml (refri.)
0	swab Campy-Cefex plate--incubate 7 h--#2 (refri.)
0	swab Campy-Cefex plate--incubate 24 h--#3 (refri.)
0	enrich remainder of suspension in Hunt's enrichment @ 30^{o}C
3	swab Campy-Cefex plate--incubate 4 h--#4 (refri.)
3	swab Campy-Cefex plate--incubate 20 h--#5 (refri.)
3	10 ml enrichment broth centrifuged--#6 (refri.)
3	add additional cefoperazone, increase temp to 37^{o}C
5	swab Campy-Cefex plate--incubate 18 h--#7 (refri.)
5	10 ml enrichment broth centrifuged--#8 (refri.)
5	continue enrichment, increase temp to 42^{o}C
24	swab Campy-Cefex plate--incubate 7 h--#9 (refri.)
24	swab Campy-Cefex plate--incubate 24 h--#10
24	Remainder of enrichment broth centrifuged--#11 (refri.)
48	Positive control--#12
48	Negative control--#13

Analyze all 11 Gene-Trak samples after #10 sample has finished incubating. At each Gene-Trak sampling, streak the same samples onto Campy-Cefex plates and incubate under microaerobic atmosphere at 42^{o}C.

Table 2. Relation of DNA probe detection (absorbance signal) to numbers of *Campylobacter jejuni* added to chicken fecal suspensions.

TRIAL 1:

strain/sample	# Campylobacter/test well	fecal sample	absorbance
negative control	none	no	0.01
positive control	none	no	0.80
none	none	#1	0.05
none	none	#2	0.01
B41	3.0×10^6	#1	0.12
	3.0×10^7		1.01
	3.0×10^8		0.86
B41	3.0×10^6	#2	0.14
	3.0×10^7		0.33
	3.0×10^8		1.14
B41	3.0×10^6	none	0.29
	3.0×10^7		0.59
	3.0×10^8		1.22
B45	2.2×10^6	#1	0.08
	2.2×10^7		0.07
	2.2×10^8		0.22
B45	2.2×10^6	#2	0.03
	2.2×10^7		0.10
	2.2×10^8		0.27
B45	2.2×10^6	none	0.03
	2.2×10^7		0.12
	2.2×10^8		0.23

TRIAL 2:

strain/sample	# Campylobacter/test well	fecal sample	absorbance
negative control	none	no	0.04
positive control	none	no	1.08
none	none	#3	0.00
none	none	#4	0.00
B41	1.6×10^6	#3	0.03
	1.6×10^7		0.26
	1.6×10^8		1.18
B41	1.6×10^6	#4	0.03
	1.6×10^7		0.53
	1.6×10^8		1.10
B41	1.6×10^6	none	0.10
	1.6×10^7		0.66
	1.6×10^8		1.37
B45	1.4×10^6	#3	0.04
	1.4×10^7		0.30
	1.4×10^8		1.10
B45	1.4×10^6	#4	0.03
	1.4×10^7		0.21
	1.4×10^8		1.15
B45	1.4×10^6	none	0.09
	1.4×10^7		0.54
	1.4×10^8		1.31

Table 3. Carcass sampling procedure and detection of *Campylobacter jejuni* by GENE-TRAK DNA probe assay and cultural methodologies.

Sampling point[1]	Chicken Number					
	Carcass samples spiked with ca 10^{2-3} cfu *C. jejuni*				no added C. jejuni	
	1	2	3	4	5	6
1	0.04	0.05	0.00	0.07	0.03	0.01
2	0.03	0.02	0.01	0.04	0.01	0.03
3	1.25*	0.03	0.02	0.07	0.02	0.01
4	0.01	0.03	0.00	0.04	0.01	0.00
5	1.16*	0.02	0.00	0.03	0.01	0.01
6	0.03	0.02	0.02	0.08	0.02	0.03
7	0.86*	0.04	0.02	0.05	0.02	0.03
8	0.02	0.03	0.00	0.07	0.04	0.04
9	0.54*	0.02	0.02	0.42*	0.03	0.00
10	0.92*	0.36*	0.51*	1.19*	0.42*	0.04
11	1.74*	0.64*	0.54*	ND	0.09	0.18*
12	1.39	0.50	0.32	0.32	0.50	0.32
13	0.05	0.02	0.05	0.05	0.02	0.05

*denotes positive cultural detection
[1]refers to the sampling points indicated in Table 1

able 4. **Identification of *Campylobacter* spp. by bacteriological culture and GENE-TRAK DNA probe nalysis of chicken carcasses.**

arcass #	Cultural Detection	Gene Trak Analysis Sample point #11	Sample point #10
1	+	-0.03	0.16
2	-	-0.01	0.00
3	+	0.05	0.95
4	-	0.50	ND
5	+	0.04	0.85
6	+	0.72	0.22
7	+	-0.02	0.71
8	+	-0.01	0.00
9	+	-0.02	0.75
10	-	1.06	ND
11	+	-0.02	0.03
12	-	0.01	ND
13	+	0.02	0.18
14	-	0.18	ND
15	-	0.18	ND
16	-	0.16	ND
17	+	0.05	0.76
18	-	0.08	ND
19	+	0.04	1.07
20	-	0.07	ND
21	-	0.06	ND
22	+	0.08	0.19
23	+	0.05	0.77
24	+	0.04	0.05
25	+	0.05	0.54
26	+	0.08	0.41
27	+	0.06	0.65
28	+	0.05	0.47
29	-	-0.01	-0.05
30	+	-0.05	-0.03
31	+	-0.05	-0.04
32	+	-0.04	-0.03
33	-	-0.03	-0.03
34	-	-0.03	-0.03
35	-	-0.02	-0.04
36	+	-0.03	0.58
37	+	-0.01	-0.02
38	-	-0.02	-0.01